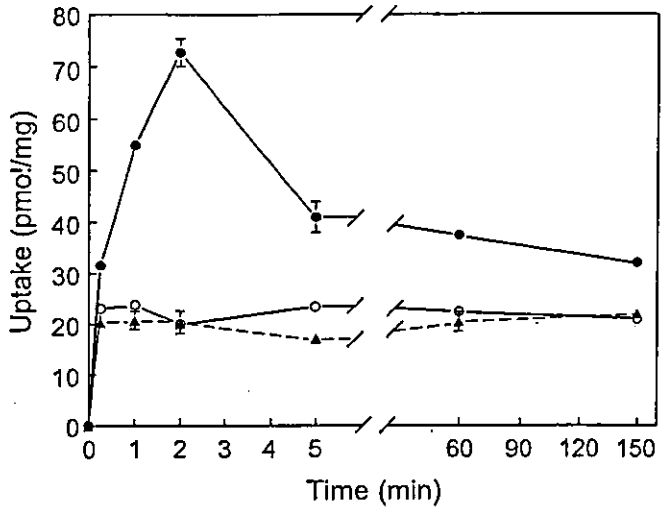

Fig. 5. Stimulatory effect of extracellular sodium and glutarate on the uptake of 2,4-dichlorophenoxyacetate by the brush border membrane of the bovine CP. Vesicles contained 100 mM mannitol, 100 mM KCl, 1 mM $MgSO_4$, 20 mM Tris–HEPES, pH 7.4. They were diluted 10-fold with transport buffer containing 10 μM [^{3}H]2,4-dichlorophenoxyacetate and either 100 mM NaCl (○) or 100 mM NaCl plus 20 μM glutarate (●). The effect of 500 μM probenecid was tested in the presence of 100 mM NaCl plus 20 μM glutarate in the external buffer (▲). Means±S.E., n=3. Taken from Pritchard et al. [142].

and glutarate at the BBM of the CP. Therefore, an outward concentration gradient of dicarboxylate produced by a sodium-dependent dicarboxylate transporter can serve as a driving force for the transporter responsible for 2,4-D uptake by the CP. 2,4-D is a poor substrate of rOat3 [60], but whether rOat3 accounts for the uptake of 2,4-D by the isolated CP remains to be examined.

The transporter involved in the basolateral excretion of hydrophilic organic anions remains unknown. Breen et al. [143] have characterized the subsequent basolateral excretion mechanism of fluorescein using confocal microscopy. They quantified the amount of fluorescein associated with the intracellular compartment and the basolateral side and demonstrated that substitution of K^+ for Na^+ reduced the accumulation of fluorescein in the basolateral compartment without affecting the amount associated with the intracellular compartment, indicating a reduction in the basolateral excretion clearance by this treatment. Since perturbation of the K^+ gradient directly or pharmacologically causes depolarization of the membrane voltage, the basolateral excretion of fluorescein is likely to be membrane voltage-dependent. The substrate specificity and molecular characteristics of this transporter remain unknown.

5. Efflux transport mechanism for organic cations in the choroid plexus

Miller and Ross [144] measured the extraction of NMN in vivo using the ventriculocisternal perfusion technique. The extraction of NMN during perfusion from the lateral ventricles to the cisternal magna was greater than that of inulin and was reduced by the addition of mepiperhenidol to the perfusate, suggesting involvement of an organic cation transporter in the extraction [144]. Other organic cations, such as cimetidine, choline, and TEA, typical substrates of renal organic cation transporters, have been shown to undergo carrier-mediated uptake at the brush border surface of the CP. The transporters for organic cations expressed in the CP are illustrated in Fig. 6.

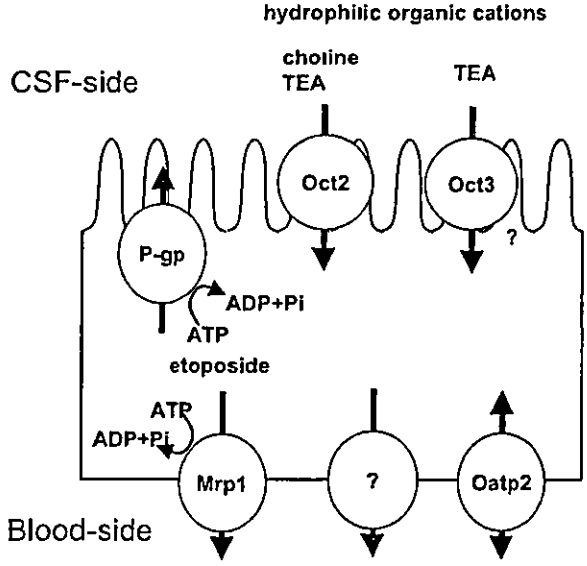

Fig. 6. Schematic diagram of the efflux transport systems for organic cations in the CP. The uptake of choline at the BBM of the CP has been suggested to be mediated by Oct2, although its expression in the CP is controversial. In addition, Oct3 mRNA was shown in the CP, but its membrane localization in the CP remains unknown. P-glycoprotein shows vesicular compartment localization in the CP, especially in the subapical region and its role in the CP remains unclear. The excretion mechanisms across the BLM remain unknown. Mrp1 has been suggested to be involved in the excretion of etoposide across the basolateral membrane. Oatp2 accepts type II organic cations as substrates, as well as amphipathic organic anions, and it may be involved in their uptake/excretion across the BLM.

5.1. Cimetidine

Cimetidine is a histamine H_2 receptor antagonist and a weak base. It is a bi-substrate, which is recognized by both organic anion and cation transporters. Ullrich et al. [145] examined the inhibitory potency of a series of compounds on the renal uptake of PAH and NMN using the stop-flow peritubular capillary microperfusion method and found that H_2 receptor antagonists, such as cimetidine, famotidine, and ranitidine, inhibit both organic anion and cation systems. It has been shown that saturable a mechanism accounts for the CSF elimination of cimetidine after i.c.v. injection [146]. Suzuki et al. [147] demonstrated saturable uptake of cimetidine by the isolated rat CP (K_m 53 μM). Organic anions, such as PAH and benzylpenicillin, produced a significant inhibition as did organic cations, such as histamine, creatinine, quinidine and quinine, while compounds, such as NMN, choline or TEA, had only a minimal effect even at 20 mM (Fig. 7) [147]. These results suggest the involvement of an organic anion transporter, rather than an organic cation transporter, as far as the uptake of cimetidine by the CP is concerned. Taking into consideration the fact that other H_2 receptor antagonists, such as famotidine and ranitidine, have been classified as bisubstrates [145], their uptake by the CP may be accounted for by the same transporter. Since cimetidine is a good substrate of rOat3 with a K_i value similar to its K_m previously determined in the CP [55,57,147], rOat3 is one of the candidates.

5.2. Tetraethylammonium and choline

Villalobos et al. [148] examined the involvement of a transporter in the uptake of TEA by isolated and primary cultured CP through the BBM of the CPE. The uptake of TEA by the isolated rat CP and primary cultured CPE was inhibited by tetrapentylammonium (TePA) (Fig. 8). Furthermore, the uptake of TEA by primary cultured cells was saturable with a K_m value of 350 μM and inhibited by NMN, darstine, choline and cimetidine, but not by PAH (Fig. 9). A preloading of unlabeled TEA into the cells prior to starting the uptake experiment stimulated the uptake of TEA by

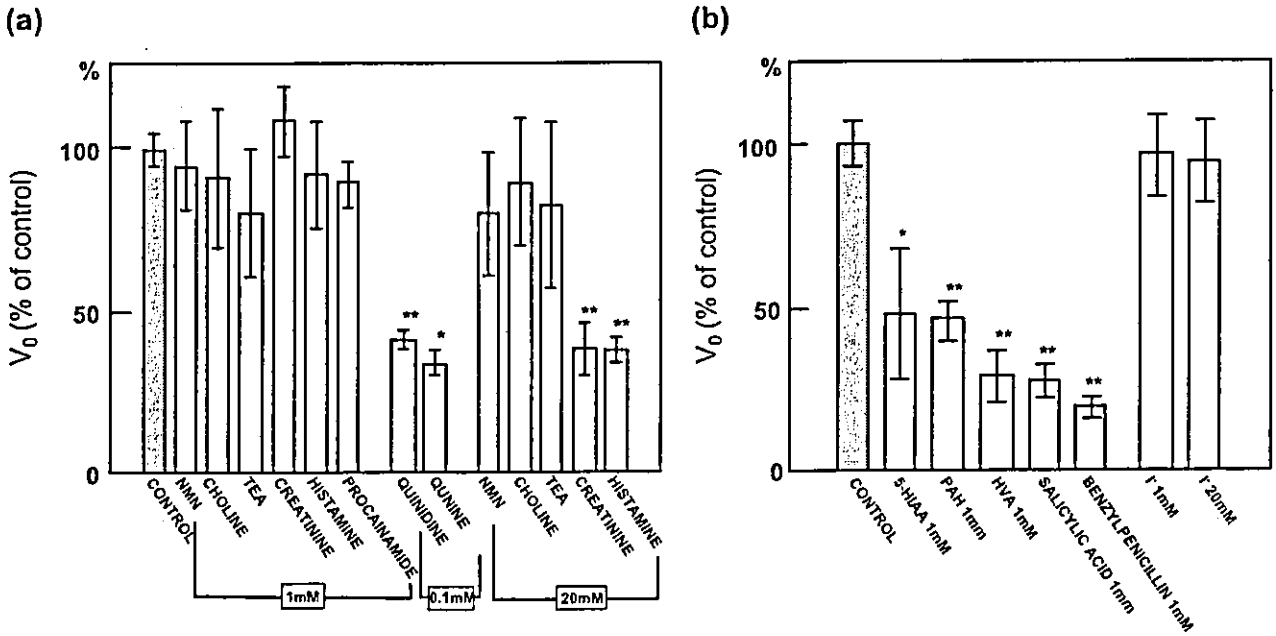

Fig. 7. Effect of organic cations and anions on the uptake of cimetidine by the isolated rat CP. [^{3}H]Cimetidine was incubated with various inhibitors at the concentrations indicated for 3 min at 37 °C. Although cimetidine is a weak base, it has been shown to be a bisubstrate which is recognized by both organic anion and cation transporters. The uptake by the isolated CP was inhibited by organic anions, such as PAH and benzylpenicillin, suggesting an involvement of an organic anion transporter, but minimal inhibition by TEA suggests a minor contribution of an organic cation transporter. Each bar represents the mean±S.E. of three independent experiments. $^{**}P<0.01$; $^{*}P<0.05$, by student's t-test. 5-HIAA, 5-hydroxyindoleacetate; HVA, homovanillic acid. Taken from Suzuki et al. [147].

primary cultured cells, suggesting that the transporter responsible for TEA uptake is characterized by its bidirectional nature.

Villalobos et al. [149] characterized the uptake mechanism using primary cultured CPE, in which a saturable uptake of choline was observed with a K_m value of 50 μM. It has been suggested that the transporter responsible for choline uptake in the primary cultured rat CPE also accepts NMN and TEA as substrates, since the preloading of primary cultured cells with NMN and TEA stimulated the uptake of choline 1.5-fold (*trans*-stimulation). The organic cation, hemicholinium-3, is a moderate inhibitor for choline uptake and NMN showed a weak inhibition. The choline transporter in the CP has been suggested to be a facilitative transporter, which depends on the membrane voltage. The uptake of choline was not affected by extracellular Na^+, whereas increasing the K^+ concentration markedly reduced the uptake, causing depolarization of the intracellular potential from −70 to −15 mV. This K^+ effect was further supported by the results that treatment of the primary cultured CPE with Na^+/K^+ ATPase (ouabain) or K^+ channel inhibitor (Ba^{2+}), causing perturbation of K^+ gradient pharmacologically, reduced the choline uptake to a degree similar to that produced by a high K^+ buffer.

Sweet et al. [71] carried out further characterization of choline uptake in the CP. According to the RT-PCR analysis by Sweet et al., both rOct2 and rOct3 mRNA are expressed in the CP, while Choudhuri et al. [26] could observe expression of rOct1 and rOct3 mRNA in the CP. Choline uptake was only observed in rOct2-expressing oocytes with a K_m value of 440 μM and not in rOct3-expressing oocytes. The K_m value of choline for rOct2 was greater than the previously reported value by Vallalobos et al. (50 μM), but

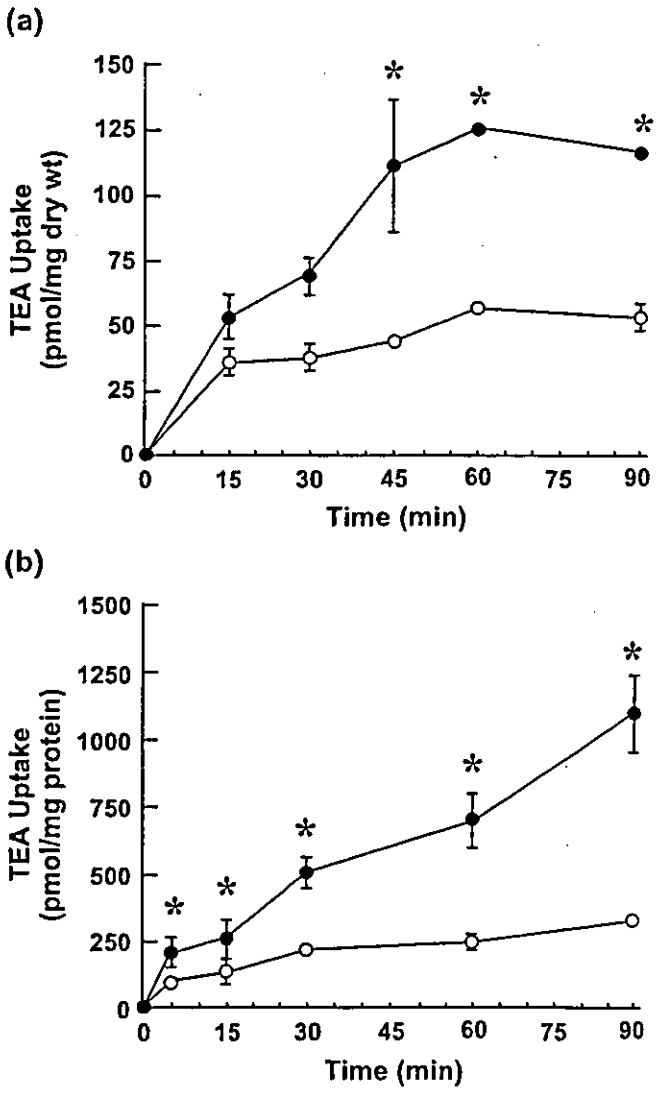

Fig. 8. Effects of TePA on the time-dependent uptake of TEA by isolated rat choroid plexus and primary cultures of rat choroid plexus epithelial cells. Isolated CP was incubated with 10 μM [^{14}C]TEA in the presence (●) or absence of tetrapentylammonium (TePA) (○, 100 μM). Similarly, cultured cells were incubated with 10 μM [^{14}C]TEA in the presence or absence of TePA (○, 1 mM). *P<0.05 vs. uptake in the absence of TePA. Taken from Villalobos et al. [148].

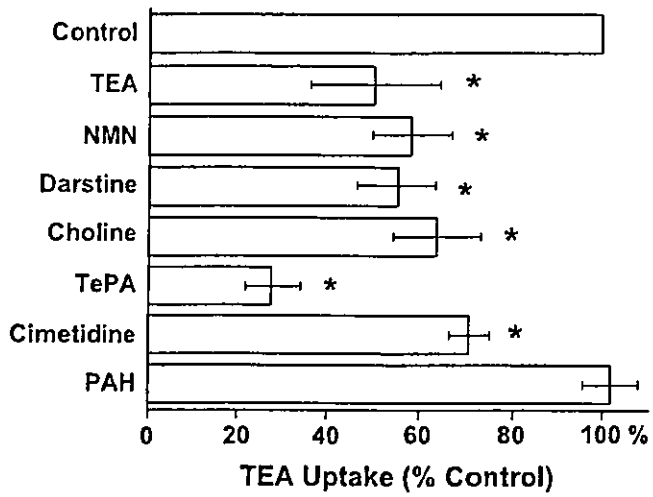

Fig. 9. Effects of organic ions on the 30 min TEA uptake by primary cultures of rat choroid plexus epithelial cells grown on a solid support. Cells were incubated with 10 μM [^{14}C]TEA in the absence (control) or presence of each inhibitor (1 mM). *P<0.05 vs. control uptake. TePA: tetrapentylammonium. Taken from Villalobos et al. [148].

comparable with the K_m value for choline uptake by the isolated rat CP, which was determined to be 180 μM in this report [71]. rOct2 may be a candidate transporter for choline uptake in the CP. Further studies are necessary to identify the localization of rOct3 in the CP and, if it is expressed at the BBM of the CP, the contribution to the uptake of hydrophilic organic cations, such as TEA, should be examined.

6. Discussion and future aspects

The present review summarizes the current status of the efflux transport mechanisms for organic ions in the CP. The many published studies have provided molecular insights into the uptake systems operating at the BBM of the CP. Due to limitations in methodology, the excretion process for organic ions has not been fully characterized yet and the molecular characteristics of the transporters involved in this process remain unknown. ABC transporters, such as MRPs and/or alternatively membrane voltage-sensitive mechanisms, are current candidates to account for this process for organic anions, while OCTNs may be involved in the excretion of hydrophilic organic cations as proposed in the kidney [82,90]. Gene knockout of the candidate gene will give us insight into the basolateral excretion of organic ions.

Evidence from many studies has shown that the primary cultured CPE retains the efflux transport systems for organic anions and cations. In addition, Kitazawa et al. have developed an immortalized cell line of rat CPE which is prepared from a transgenic rat harboring the temperature-sensitive SV40 large T antigen [24,150,151]. These models will allow us to investigate the transport mechanisms for small compounds across the monolayer of the CPE. It is necessary to compare the expression levels of transporter genes in these models with those in freshly isolated CP in future studies. RNA interference has been developed and shown to efficiently suppress target genes even in mammalian cells [152]. This new methodology will help us to investigate the transport mechanisms in the CP from a genetic viewpoint.

Once a transporter involved in the efflux transport has been identified, cDNA-transfectants expressing human transporters serve as a screening system for the selection of drugs with suitable pharmacokinetic properties. Recently, Sasaki et al. [153] and Cui et al. [154] established double transfectants, which express uptake and efflux transporters (OATP-C or OATP 8, and MRP2, respectively) in the basal and apical membrane of MDCK II cells, respectively. The basal-to-apical transport of their common substrates is greater in OATP-C/MRP2 or OATP8/MRP2 than that in control or single gene transfected cells and this transport mimics the hepatobiliary transport in humans [153,154]. The same approach can be used as an in vitro model of the CPE after the transporters involved in the uptake and efflux have been identified. Further studies are necessary to discover whether human orthologs of the transporters identified in the rodent CP are expressed in the human tissues. Furthermore, the interindividual differences in the transport activities due to up- or down-regulation in disease states and genetic polymorphisms are important topics which also need to be investigated in future studies.

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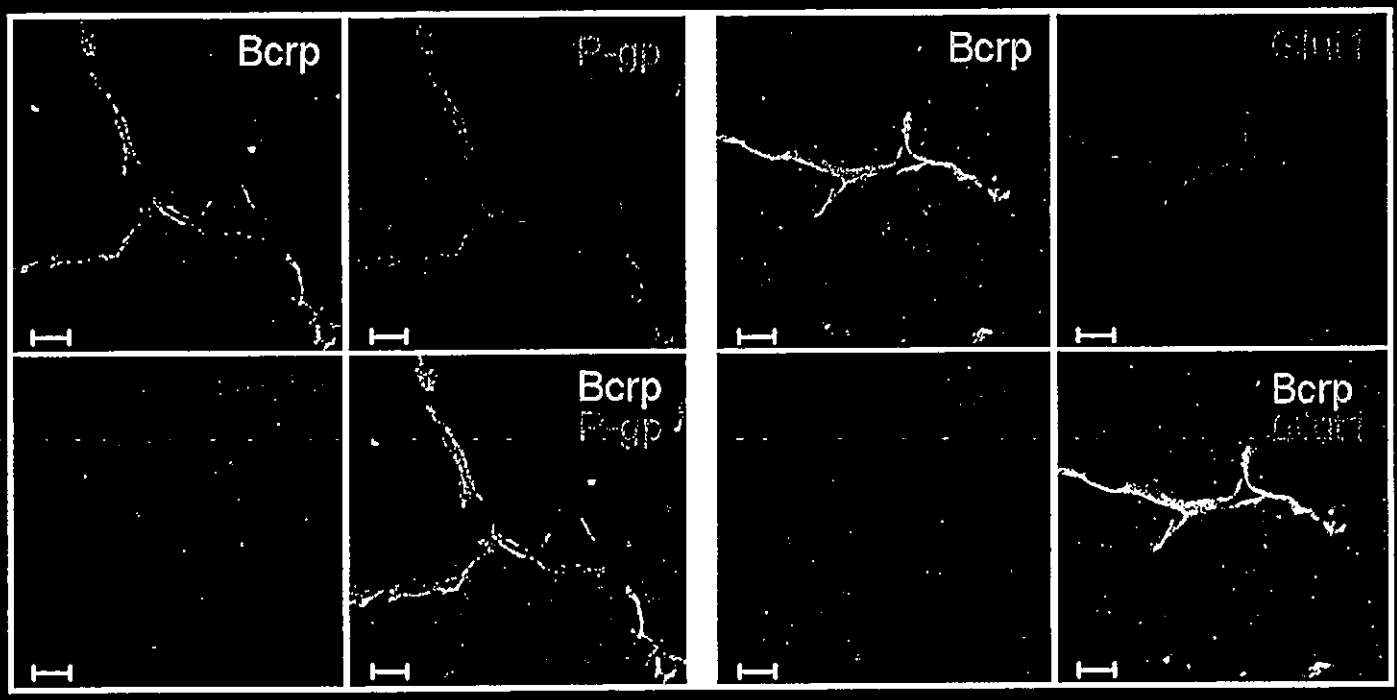

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Investigation of Efflux Transport of Dehydroepiandrosterone Sulfate and Mitoxantrone at the Mouse Blood-Brain Barrier: A Minor Role of Breast Cancer Resistance Protein

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ABSTRACT

Breast cancer resistance protein (Bcrp/Abcg2) is a new efflux transporter found at the blood-brain barrier (BBB) of humans and pigs. Since it has been hypothesized that Bcrp may act as a new type of efflux transporter at the BBB, we investigated the involvement of Bcrp in the efflux transport of typical substrates, dehydroepiandrosterone sulfate (DHEAS) and mitoxantrone, across the mouse BBB. The expression of Bcrp in mouse brain capillaries was confirmed by quantitative polymerase chain reaction, Western blot, and immunohistochemical analysis. The role of Bcrp as an efflux transporter was evaluated using the in situ brain perfusion method in wild-type and P-glycoprotein (P-gp) knockout mice with or without treatment with GF120918 (Elacridar), an inhibitor of both Bcrp and P-gp. The increased brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone by GF120918 in wild-type and P-gp knockout mice suggested the existence of a GF120918-sensitive and P-gp-independent efflux transporter for DHEAS and mitoxantrone across the BBB. However, the brain uptake of [^{3}H]DHEAS in Bcrp knockout mice was comparable with that in wild-type mice, and the effect of GF120918 was still observed in Bcrp knockout mice. In addition, the brain uptake of [^{3}H]mitoxantrone was also similar in wild-type and Bcrp knockout mice. These results suggest that although BCRP is expressed at the BBB it plays a minor role in active efflux transport of DHEAS and mitoxantrone out of brain and that one or more GF120918-sensitive efflux transporters distinct from BCRP or P-gp contributes to the brain efflux of DHEAS and mitoxantrone.

It is well known that the transport of compounds from the circulating blood into the central nervous system is restricted by the blood-brain barrier (BBB), which is formed by the brain capillary endothelial cells that are characterized by highly developed tight junctions and a paucity of fenestra and pinocytotic vesicles. In addition to these characteristics, efflux transporters expressed in the brain capillaries play an important role in the elimination of endogenous waste products and xenobiotics from the brain and prevent their accumulation in the central nervous system (Kusuhara and Sugiyama, 2001; Sun et al., 2003). Breast cancer resistance protein (BCRP/*ABCG2*) is a unique ABC efflux transporter that accepts sulfoconjugated organic anions as well as hydrophobic and amphiphilic compounds as substrates. Bcrp has been shown to restrict the intestinal absorption and fetal penetration of its substrates, such as mitoxantrone and topotecan (Allen et al., 1999; Jonker et al., 2002; Kruijtzer e al., 2002). BCRP has also been found at the luminal side o human and porcine brain capillary endothelial cells (Cooray et al., 2002; Eisenblatter and Galla, 2002; Zhang et al., 2003). Overexpression of human BCRP in immortalized rat brain endothelial cells resulted in enhanced vectorial transport of mitoxantrone, fluorescein, and rhodamine-123 in the abluminal-to-luminal direction (Zhang et al., 2003). Recently, overexpression of Bcrp in multidrug-resistance (Mdr)1a P-glycoprotein (P-gp) knockout mice has also been reported (Cisternino et al., 2004). However, it is unclear whether

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ABBREVIATIONS: BBB, blood-brain barrier; BCRP, breast cancer resistance protein; Mdr, multidrug-resistance; P-gp, P-glycoprotein; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; OAT, organic anion transporter(s); OATP, organic anion-transporting polypeptide; GF120918, Elacridar; RT-PCR, reverse transcriptase-polymerase chain reaction; HPRT, hypoxanthine phosphoribosyl-transferase; Mrp, multidrug resistance-associated protein; TBST, Tris-buffered saline/Tween 20; PBS, phosphate-buffered saline; Glut, glucose transporter; BSA, bovine serum albumin; ANOVA; analysis of variance.

BCRP plays a role as an efflux transporter at the luminal membrane of the brain capillaries in vivo, together with P-gp (Schinkel et al., 1994, 1996).

As a neurosteroid, dehydroepiandrosterone sulfate (DHEAS) and its unconjugated form (DHEA) modulates neurotransmission in an excitatory or inhibitory manner via ion-gated channels involving *N*-methyl-D-aspartate receptors and γ-aminobutyric acid receptors (Schumacher et al., 1997). In rodents, DHEAS and DHEA are also synthesized locally in the brain, and these neurosteroids can be interchanged via sulfotransferase and sulfatase (Baulieu, 1996; Stoffel-Wagner, 2001), and the level of DHEAS is much higher than that of DHEA in the brain and plasma of rats (Corpechot et al., 1981). At least in rodents, a compartmental barrier is known to exist for DHEAS between the brain and circulating blood (Biggio and Purdy, 2001), and in humans, the concentration of DHEAS in brain is known to be much lower than that in blood (Weill-Engerer et al., 2002).

DHEAS is a substrate of several transporters, including Na^+-taurocholate cotransporting polypeptides, organic anion-transporting polypeptides (Oatp/OATP), and organic anion transporters (Oat/OAT) (Kullak-Ublick et al., 1998; Hagenbuch and Meier, 2003; Hasegawa et al., 2003). In particular, Oatp2 (*Slco1a4*, OATP1a4) has been reported to be a candidate efflux transporter for DHEAS at the BBB (Asaba et al., 2000). Interestingly, Asaba et al. also suggested the existence of primary active efflux transporter(s) for DHEAS in a conditionally immortalized cell line established from mouse brain capillary endothelial cells (TM-BBB4). The net uptake of DHEAS by TM-BBB4 was increased under ATP-depleted conditions (Asaba et al., 2000). This efflux transport system is expected to account for the compartmentalization of DHEAS between brain and the circulating blood and also to be one of the mechanisms of inactivation of DHEAS in the brain to regulate its activity on neurons.

Our previous study using membrane vesicles prepared from BCRP overexpressed P-388 cells clearly showed that DHEAS is an endogenous substrate of BCRP (Suzuki et al., 2003). Therefore, we hypothesized that BCRP plays a role in the luminal excretion of DHEAS at the brain capillaries as an unidentified primary active efflux transporter(s) predicted by Asaba et al. (2000). This hypothesis was partially supported by the results of Jonker et al. They showed that GF120918 (Elacridar), an acridine derivative known to be an inhibitor of P-gp and BCRP, increased the in vivo oral bioavailability and fetal penetration of topotecan even in Mdr1a/1b P-gp knockout mice, suggesting a function for BCRP in the intestinal barrier and maternal-fetal barrier (Jonker et al., 2000). In the present study, the expression and localization of Bcrp at the mouse BBB was investigated by real-time quantitative RT-PCR, Western blot analysis, and immunohistochemical staining. The brain uptake of DHEAS was determined using the in situ brain perfusion method, and the effect of GF120918 on brain uptake was examined. The brain uptake of mitoxantrone, a typical substrate of Bcrp, was also investigated in the same manner. Finally, the involvement of mouse Bcrp in the efflux of DHEAS and mitoxantrone across the BBB was evaluated directly using the recently established Bcrp knockout mouse (Jonker et al., 2002).

Materials and Methods

Reagents and Animals. [^{3}H]DHEAS was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{14}C]Sucrose and [^{3}H]mitoxantrone were purchased from Moravek Biochemicals (Brea, CA). GF120918 was a gift from GlaxoSmithKline (Uxbridge, Middlesex, UK). USP grade propylene glycol was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in the experiments were of analytic grade.

Male Mdr1a/1b P-gp knockout mice and age-matched wild-type control mice were purchased from Taconic Farms (Germantown, NY), and male Bcrp knockout mice and age-matched wild-type mice of a comparable genetic background were produced as reported (Jonker et al., 2002). All mice (8 ~ 22 weeks) were maintained under standard conditions with a reverse dark/light cycle. Food and water were available ad libitum.

Isolation of Mouse Brain Capillaries. A brain capillary-enriched fraction from the mouse brain (BBB-enriched fraction) was isolated according to the reported procedure with slight modification (Dallaire et al., 1991; Ball et al., 2002). Briefly, large brains were dissected from the heads after perfusion with 0.9% saline and homogenized using a Polytron homogenizer in a 0.32 M sucrose solution and centrifuged at 4°C at 2200*g* for 10 min. The pellet was further purified according to the procedures suggested by Dallaire et al. (1991) and used as a BBB-enriched fraction. All reagents as well as the tissue should be kept on ice or as close to 4°C as possible throughout the isolation process to minimize degradation.

The purity of the isolated BBB-enriched fraction was checked by the enhanced alkaline phosphatase activity in the brain homogenate and BBB-enriched fraction (Dallaire et al., 1991; Ball et al., 2002). Isolated brain capillary-enriched fraction from mice contained tangled skeins of microvessel, which was confirmed under light microscopy. The alkaline phosphatase activity in the BBB-enriched fraction was 18.6-fold greater than that in the brain homogenate. This BBB-enriched fraction was used for further analyses: Western blot and real-time quantitative PCR.

Quantification of Transporter mRNA in the Brain Homogenate and the BBB-Enriched Fraction. To quantify the expression of Bcrp at the mouse BBB, real-time quantitative PCR was used. Total RNA was isolated from the BBB-enriched fraction and brain homogenate from wild-type FVB mice using an RNeasy mini kit (QIAGEN, Valencia, CA) and was converted to cDNA using random primer and avian myeloblastosis virus reverse transcriptase. Real-time quantitative PCR was performed using a QuantiTect SYBR Green PCR kit (QIAGEN) and LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The primers used in the quantification are listed in Table1. All primers were designed based on the published full sequence of each

TABLE 1
Nucleotide sequences of the primers used in quantitative PCR

	Forward Primer	Reverse Primer	Gene Bank Accession No.
HPRT	GCTTTCCCTGGTTAAGCAGTACA	CAAACTTGTCTGGAATTTCAAATC	J00423
Bcrp	AAATGGAGCACCTCAACCTG	CCCATCACAACGTCATCTTG	NM_011920
Mdr1a	TCATTGCGATAGCTGGAGTG	CAAACTTCTGCTCCCGAGTC	NM_011076
Mrp1	AGGCTGGAGCTAAGGAGGAG	CAGCCATGGAGTAGCCAAAT	NM_008576
Mrp4	GGTTGGAATTGTGGGCAGAA	TCGTCCGTGTGCTCATTGAA	XM_139262
Oatp2	ATAGCTTCAGGCGCATTTAC	TTCTCCATCATTCTGCATCG	NM_030687

protein. Hypoxanthine phosphoribosyl-transferase (HPRT) was used as a housekeeping gene for the internal standards, and Mdr1a was used as a positive control gene for putative transporter at the brain microvessel (Ball et al., 2002). An external standard curve was generated by dilution of the target PCR product, which was purified by agarose gel electrophoresis. The absolute concentration of external standard was measured by PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR). To confirm amplification specificity, PCR products were subjected to a melting curve analysis and gel electrophoresis. All gene expressions in each reaction were normalized by the expression of HPRT in the same sample (Ball et al., 2002).

Besides Bcrp, the mRNA of Mdr1a, multidrug resistance-associated protein 1 (Mrp1), Mrp4, and Oatp2 in total brain cortex of wild-type and Bcrp knockout mouse were also measured by real-time quantitative PCR. These transporters have been reported to be expressed in the mouse brain, and this result was used to show the relative expression of these transporters in the wild-type mouse brain and to examine the possibility of up- and/or down-regulation of these transporters in the brain of the Bcrp knockout mouse.

Western Blot Analysis. The lysates of the BBB-enriched fraction and brain homogenate were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Immobilon; Millipore Corporation, Bedford, MA) which were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% skim milk for 2 h at room temperature. After washing with TBST, membranes were incubated with anti-BCRP monoclonal antibody (40-fold diluted BXP-53 antibody; Signet Laboratories, Dedham, MA) in TBST overnight at 4°C, and proteins were detected using the ECL system (Amersham Biosciences Inc., Arlington Heights, IL).

Immunocytochemical Analysis of the Expression of Bcrp at the Mouse BBB. Brain samples from the wild-type mouse and Bcrp knockout mouse were fixed in 4% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin according to standard procedures. For immunohistochemistry, tissues were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked by 3% (v/v) H_2O_2 in methanol for 10 min. Before staining, paraffin sections were pretreated by heat-induced epitope retrieval. Slides were incubated with 5% normal goat serum/PBS for 30 min, and subsequently, sections were incubated overnight with a 1:400 dilution of BXP-53 at 4°C. Monoclonal antibody immunoreactivity was detected by the streptavidin-biotin immunoperoxidase (sABC) method by using biotinylated goat anti-rat IgG (Dako, 1:100) as a secondary antibody and diaminobenzidine substrate for visualization. After counterstaining with hematoxylin, slides were mounted. To investigate the localization of Bcrp in brain microvessels, double immunostaining with antibodies of P-gp (luminal expression) and glucose transporter 1 (Glut1, luminal and abluminal coexpression) was also performed using cryostat sections of wild-type mouse brain (10-μm thick) (Cooray et al., 2002). Brain sections without fixation were incubated overnight at 4°C with primary antibody at the following concentrations: Bxp-53 (Bcrp, 1:40 dilution in 1% BSA/PBS), C219 (P-gp, 1:40 dilution in 1% BSA/PBS; Signet Laboratories), and anti-Glit1 (Glut1, 1:40 dilution in 1% BSA/PBS; Santa Cruz Biochemicals, Santa Cruz, CA). After washing with PBS, sections were incubated with appropriate Alexa Fluor secondary antibodies (Molecular Probes) and Topro3 (DNA dye; Molecular Probes, Hilversum, Netherlands) for 1 h and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized under a Zeiss confocal fluorescence microscope.

In Situ Brain Perfusion to Determine the Brain Uptake of Bcrp Substrates. The right cerebral hemisphere of the mouse was perfused using the reported method (Takasato et al., 1984, Dagenais et al., 2000; Murakami et al., 2000) with minor modification. In brief, the mouse was anesthetized by intraperitoneal injection of 10 mg/kg xylazine (Sigma-Aldrich) and 100 mg/kg ketamine (Sankyo Co., Tokyo, Japan). The right common carotid artery was exposed and then catheterized with polyethylene tubing (0.2 mm i.d. × 0.5 mm o.d; Natsume, Tokyo, Japan) filled with heparinized saline. The right hemisphere of the brain was perfused with Krebs bicarbonate buffer (pH 7.4 with 95% O_2 and 5% CO_2 containing 10 mM D-glucose) at a flow rate of 1 ml/min (Murakami et al., 2000). The thorax of the mouse was opened, and the cardiac ventricle was severed immediately before perfusion. [^{3}H]DHEAS or [^{3}H]mitoxantrone was added to perfusate at a concentration of 1 μCi/ml with carbon-labeled sucrose as a vascular volume marker. Perfusion was terminated by decapitation at selected times (1 and 2 min, for [^{3}H]DHEAS; 1.5 min for [^{3}H]mitoxantrone). The right hemisphere of the brain was removed from the skull and weighed. Aliquots of the perfusion fluid also were collected to determine tracer concentrations in the perfusate. Brain samples were digested in 2 ml 1 N NaOH at 55°C, and the dual radioactivity associated with the brain was measured in a liquid scintillation counter (LS 6000SE; Beckman Coulter, Fullerton, CA).

In all perfusion experiments, the brain vascular volume (V_{vasc}; microliter per gram) was estimated from the tissue distribution of [^{14}C]sucrose, which is known to diffuse very slowly across the BBB, using the following equation (Dagenais et al., 2000):

$$V_{vasc} = X_{sucrose}/C_{sucrose} \quad (1)$$

where $X_{sucrose}$ (disintegrations per minute per gram) is the amount of sucrose measured in the right brain hemisphere and $C_{sucrose}$ (disintegrations per minute per milliliter) is the concentration of labeled sucrose in the perfusion fluid. V_{vasc} is the brain vascular distribution volume of substrate used to check BBB integrity during the experiments (Dagenais et al., 2000).

Brain distributional volume of substrate (V_{brain}, microliters per gram) is calculated as:

$$V_{brain} = X_{brain}/C_{substrate} \quad (2)$$

where X_{brain} is the amount of substrate in the brain (disintegrations per minute per gram) corrected for vascular contamination ($X_{total} - V_{vasc} \times C_{substrate}$) and $C_{substrate}$ is the concentration of substrate in the perfusate (disintegrations per minute per milliliter) (Dagenais et al., 2000).

The uptake clearance of substrate (CL_{up}, microliters per gram) is calculated as the slope of the plot of time versus V_{brain}.

$$CL_{up} = X_{brain}/T/C_{substrate} \quad (3)$$

where T is the perfusion time (min) (Dagenais et al., 2000).

Effects of Bcrp and P-gp on the Brain Uptake of [^{3}H]DHEAS and [^{3}H]Mitoxantrone. As an inhibitor of Bcrp, GF120918 (10 or 20 mg/kg, dissolved in a 3:2 mixture of propylene glycol/water) was injected intravenously to mice (125 μl/25g mice) at 10 min before the in situ perfusion of [^{3}H]DHEAS and [^{3}H]mitoxantrone (Hyafil et al., 1993, Cisternino et al., 2001). Because GF120918 inhibits both P-gp and Bcrp (Allen et al., 1999), the role of Bcrp on the brain uptake of substrates was investigated by comparing the brain uptake in wild-type control mice and P-pg knockout mice with or without treatment with GF120918, respectively, to exclude any confounding effects of P-gp inhibition (Jonker et al., 2000). The role of Bcrp on BBB transport was also examined directly by comparing the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone in Bcrp knockout mice and wild-type control mice (Jonker et al., 2002). Control groups received only vehicle solution in all experiments.

Statistical Analysis. Data are presented as the mean ± standard error for 3 to 10 animals unless specified otherwise. Student's two-tailed unpaired t test and one-way ANOVA followed by the Newman-Keuls multiple comparison test were used to identify significant differences between groups when appropriate. Statistical significance was set at $p < 0.05$.

Results

The Expression of Bcrp at the Mouse BBB. The expression of Bcrp at the BBB was suggested by comparing

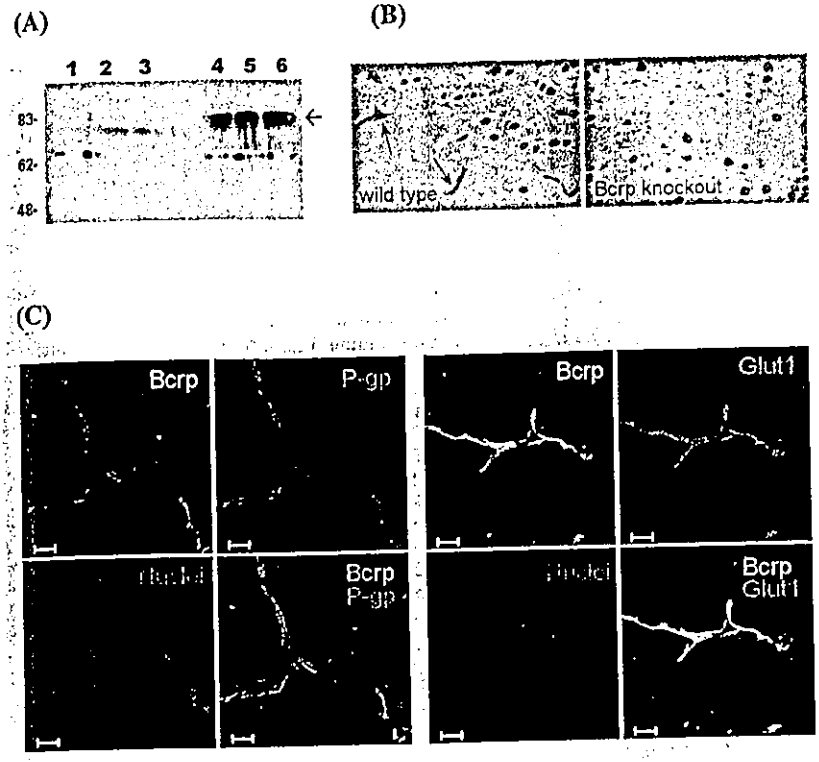

Fig. 1. Expression of Bcrp at the BBB. A, Western blot of brain homogenate and the BBB-enriched fraction with anti-mouse BCRP monoclonal antibody (BXP-53). Approximately 70 kDa of Bcrp band in BBB-enriched fraction was clearly detected (lanes 4–6), whereas only faint bands were observed in brain (lanes 1–3). Lane 1, 4 (25 μg of protein); lane 2, 5 (50 μg of protein); lane 3, 6 (100 μg of protein). B, immunohistochemical detection of Bcrp in brain sections from wild-type (left) and Bcrp knockout mice (right). A consistent staining of blood capillaries for Bcrp (brown) throughout the mouse brain was observed, suggesting moderate Bcrp expression in wild-type brain capillaries, whereas it was completely absent in the Bcrp knockout mouse brain. C, double immunostaining of Bcrp with P-gp and Glut1. P-gp was used as a marker of luminal expression and Glut1 was used as a marker of luminal and abluminal coexpression: green, Bcrp; red, P-gp (left) or Glut1 (right); blue color indicates nuclei stained with Topro3. The Bcrp and P-gp signals were completely superimposed, whereas the Bcrp signal only partially overlapped with Glut1 signals, suggesting luminal expression of Bcrp in brain capillaries. Scale bar = 10 μm.

mRNA expression between brain homogenate and capillary-enriched fraction by real-time quantitative PCR. The concentration of Bcrp mRNA, which was normalized by that of HPRT, was 5.6-fold higher in the BBB-enriched fraction than that in brain homogenate (5.6 ± 1.3, mean ± S.D.), whereas that of Mdr1a mRNA was enriched 12-fold in the BBB-enriched fraction (12 ± 2, mean ± S.D.).

The protein band (70 kDa) of Bcrp in the BBB-enriched fraction was clearly detected by Western blot analysis using monoclonal antibody BXP-53, whereas only weak staining was observed in the brain homogenate, suggesting that the primary localization of Bcrp is at the BBB (Fig. 1A). Furthermore, immunohistochemical analysis of brain sections of wild-type and Bcrp knockout mice showed Bcrp expression only in the brain capillaries of the wild-type mouse (Fig. 1B). Although the absolute staining level does not appear to be very high, Bcrp-related staining of blood capillaries throughout the mouse brain was clearly observed, whereas it was completely absent in Bcrp knockout mouse brain (Fig. 1B). Double immunostaining of Bcrp with P-gp and Glut1 clearly showed that Bcrp is expressed at the luminal side of brain microvessels (Fig. 1C). The Bcrp signal was completely superimposed on that of P-gp expressed at the luminal side of the brain microvessels. However, the Bcrp signal only partially overlapped with that of Glut1 expressed at the abluminal and luminal side of the brain microvessel.

The Effect of Pretreatment with GF120918 on the Brain Uptake of [^{3}H]DHEAS and [^{3}H]Mitoxantrone. The time-dependent brain uptake of [^{3}H]DHEAS in mice is shown in Fig. 2. The brain uptake increased linearly, and the uptake clearances could be calculated from the slope of the plot of V_{brain} versus time (Dagenais et al., 2000). The brain uptake clearance of [^{3}H]DHEAS was 18.0 μl/min/g of brain (Fig. 2). Pretreatment of wild-type mice with GF120918 (10 mg/kg) increased the brain uptake clearance of [^{3}H]DHEAS about 2.1-fold (approximately estimated, 38.6 μl/min/g of brain). Increasing the dose of GF120918 (20 mg/kg) showed a further increase in the brain uptake of [^{3}H]DHEAS by 3.0-fold (approximately estimated, 54.0 μl/min/g of brain) (Fig. 2). The V_{brain} of [^{3}H]DHEAS at 2 min was increased 2.0- and 2.8-fold by pretreatment with GF120918 10 and 20 mg/kg, respectively (Fig. 2; *, $p < 0.05$).

The brain uptake of [^{3}H]DHEAS (Fig. 3A) in Mdr1a/1b P-gp knockout mice was comparable with that in wild-type mice. However, GF120918 (10 mg/kg) increased the brain uptake of [^{3}H]DHEAS even in the Mdr1a/1b P-gp knockout

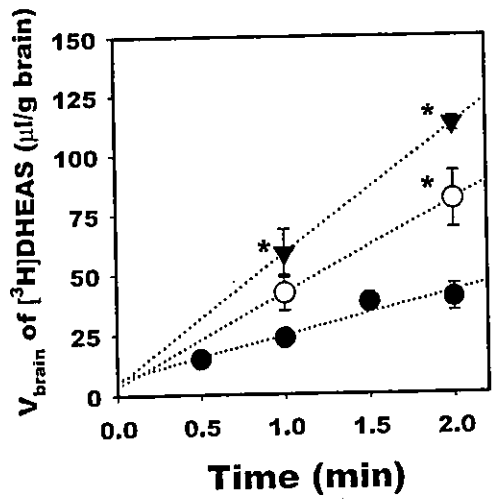

Fig. 2. Time-dependent and GF120918-sensitive brain uptake of [^{3}H]DHEAS. Brain uptake is expressed as brain distribution volume, V_{brain} and uptake clearances could be calculated by the slope of plot of V_{brain} versus time profile. ●, control, CL_{up} = 18.0 μl/min/g of brain; ○, +GF120918, 10 mg/kg, CL_{up} = 38.6 μl/min/g of brain; ▼, +GF120918, 20 mg/kg, CL_{up} = 54.0 μl/min/g of brain (n = 3 ~ 10 per point; *, statistically different with control, $p < 0.05$).

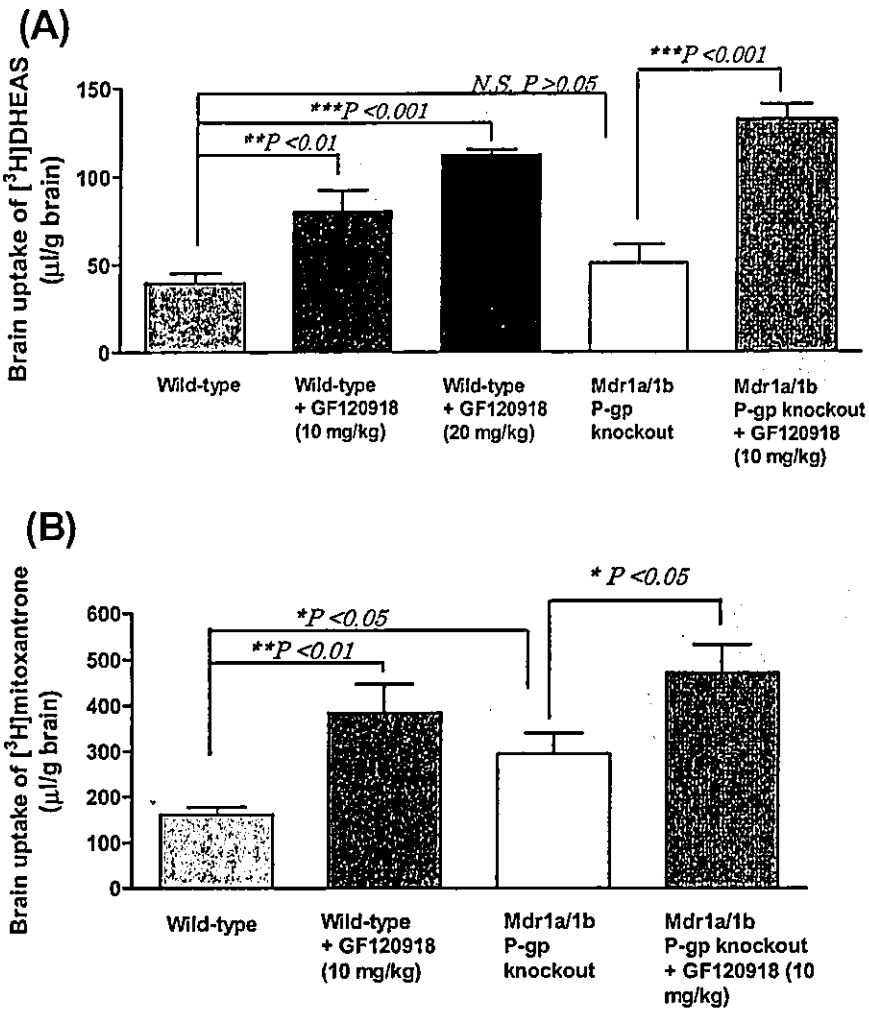

Fig. 3. Brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone, which was expressed as brain distribution volume, V_{brain}. A, the GF120918-sensitive brain uptake of [^{3}H]DHEAS at 2 min after in situ brain perfusion (V_{brain} at 2 min). The pretreatment of GF120918 increased the brain uptake of [^{3}H]DHEAS, whereas knockout of Mdr1a/1b did not. Data are presented as the mean ± S.E. (n = 3 ~ 10 per point). B, GF120918-sensitive brain uptake of [^{3}H]mitoxantrone at 1.5 min after in situ brain perfusion (V_{brain} at 1.5 min). A knockout of Mdr1a/1b and the pretreatment of GF120918 increased the brain uptake of [^{3}H]mitoxantrone. The pretreatment of GF120918 increased the brain uptake of [^{3}H]mitoxantrone even in Mdr1a/1b P-gp knockout mice. Data are presented as the mean ± S.E. (n = 4 ~ 6 per point). Statistical significance was calculated by one-way ANOVA followed by Newman-Keuls multiple comparison tests.

mice (Fig. 3A). The V_{brain} of [^{3}H]DHEAS at 2 min was increased 2.6-fold by pretreatment with GF120918 (10 mg/kg) in Mdr1a/1b P-gp knockout mice compared with that in the nontreated group.

The brain uptake of [^{3}H]mitoxantrone in wild-type mice and Mdr1a/1b P-gp knockout mice, with or without pretreatment with GF120918, is presented in Fig. 3B. The brain uptake of [^{3}H]mitoxantrone was increased in Mdr1a/1b P-gp knockout mice (1.8-fold) and wild-type mice following pretreatment with GF120918 (10 mg/kg, 2.4-fold). GF120918 (10 mg/kg) also increased the brain uptake of [^{3}H]mitoxantrone in Mdr1a/1b P-gp knockout mice (1.6-fold). The inhibitory effect of GF120918 was found to be more potent in Mdr1a/1b P-gp knockout mice for [^{3}H]DHEAS (Fig. 3A, wild-type + GF120918, 10 mg versus Mdr1a/1b P-gp knockout + GF120918, 10 mg; $p < 0.05$ Newman-Keuls multiple comparison test), although the reason was unclear. In the case of [^{3}H]mitoxantrone, no significant difference was noted between the wild-type + GF120918 10-mg group and the Mdr1a/1b P-gp knockout + GF120918 10-mg group.

Effect of Bcrp Gene Knockout on the Brain Uptake of [^{3}H]DHEAS and [^{3}H]Mitoxantrone. The impact of Bcrp on the transport of [^{3}H]DHEAS and [^{3}H]mitoxantrone across the BBB was evaluated directly using the Bcrp gene knockout mouse (Fig. 4, A and B). The brain uptake of [^{3}H]DHEAS in Bcrp knockout mice was not different from that in wild-type mice (Fig. 4A). Pretreatment with GF120918 (10 mg/kg) increased the brain uptake of [^{3}H]DHEAS even in Bcrp knockout mice. The V_{brain} of [^{3}H]DHEAS at 2 min was increased about 1.8- and 2.2-fold by GF120918 (10 mg/kg) in wild-type and Bcrp knockout mice, respectively.

In addition, the brain uptake of [^{3}H]mitoxantrone in Bcrp knockout mice was no different from that in wild-type mice (Fig. 4B). The V_{brain} of [^{3}H]mitoxantrone at 1.5 min was 144 ± 3 μl/g brain (n = 3) in the wild-type mouse and 161 ± 18 μl/g brain (n = 3) in the Bcrp knockout mouse, respectively, and there was no statistical difference between the two groups (unpaired Student's t test, $p > 0.05$).

Assessment of BBB Integrity. In all experiments, the physical integrity of the BBB was assessed by [^{14}C]sucrose, which serves as a brain vascular space marker. The brain vascular space under each set of experiment conditions was not changed by knockout of the Mdr1a/1b gene and pretreatment with GF120918 (Table 2). A similar brain vascular space was also observed between Bcrp knockout mice and their wild-type controls suggesting that the BBB integrity was not adversely affected by these experiments.

Relative Expression of Transporters in Mouse Brain. To estimate the relative expression of these transporters in the wild-type mouse brain and to examine the possibility of up- and/or down-regulation of these transporters in the brain of the Bcrp knockout mouse, mRNA quantification of known transporters such as Mdr1a, Mrp1, Mrp4, and Oatp2 was carried out using cDNA prepared from mouse brain homog-

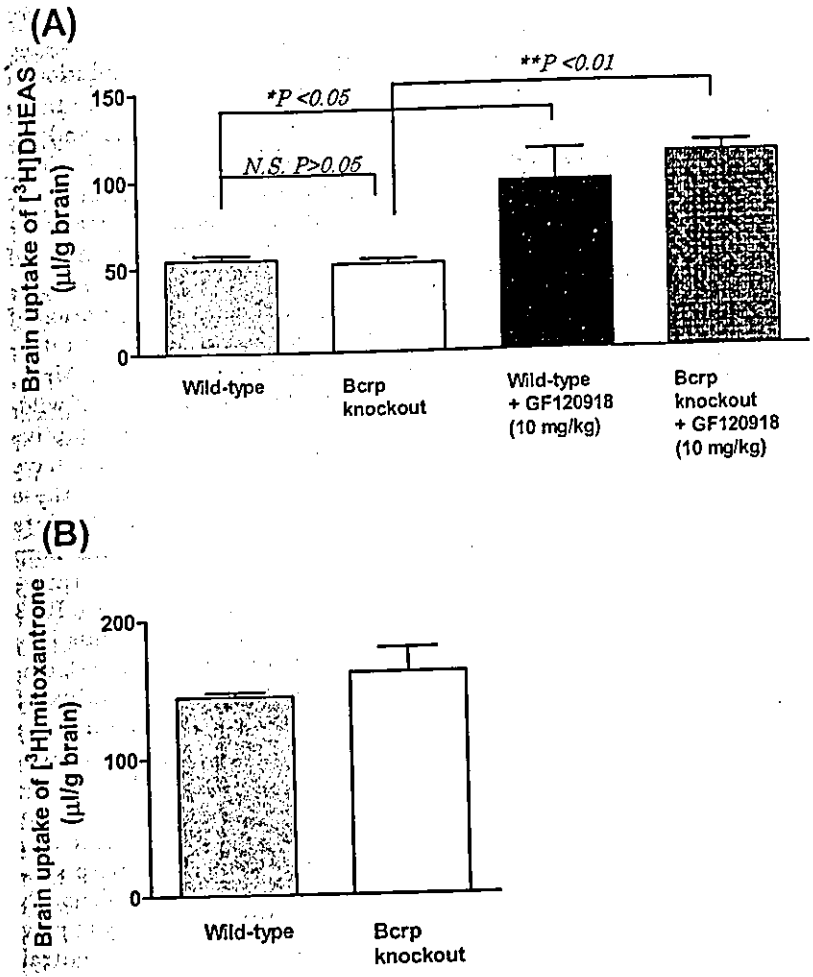

Fig. 4. Brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone in Bcrp knockout mice. A, the brain uptake of [^{3}H]DHEAS in Bcrp knockout mice was not different with that in wild-type control mice of the same genetic background, and the brain uptake of [^{3}H]DHEAS was increased by treatment of GF120918 in wild-type mice and even in Bcrp knockout mice. Data are presented as the mean ± S.E. (n = 3, respectively). Statistical significance was calculated by one-way ANOVA followed by Newman-Keuls multiple comparison tests. B, brain uptake of [^{3}H]mitoxantrone. The brain uptake of [^{3}H]mitoxantrone in Bcrp knockout mice was not different with that in wild-type control mice. Data are presented as the mean ± S.E. (n = 3, respectively). Statistical significance was calculated by unpaired t test.

TABLE 2

Vascular volume (V_{vasc}, microliters per gram of brain) of mouse brain in each experiment

Vascular volume was determined by coperfusion of [^{14}C]sucrose for 2 min ([^{3}H]DHEAS) or 1.5 min ([^{3}H]mitoxantrone). Vascular volumes did not differ significantly between any of the experiments ($p > 0.05$, one-way ANOVA). Data are presented as the mean ± S.E. ($n = 4 \sim 10$ per point).

	Wild-Type Mice			Mdr1a/1b P-gp Knockout Mice		Bcrp Knockout Mice	
Vascular Volume	Control	GF120918		Control	GF120918 (10 mg/kg)	Control	GF120918 (10 mg/kg)
		10 mg/kg	20 mg/kg				
μl/g brain							
DHEAS	12.8 ± 1.2	16.9 ± 1.9	15.5 ± 0.8	11.3 ± 0.8	12.4 ± 0.5	11.3 ± 0.5	8.96 ± 1.26
Mitoxantrone	11.9 ± 2.6	11.3 ± 4.3		16.6 ± 1.8	16.7 ± 4.3	12.9 ± 1.3	13.0 ± 1.6

enate (Table 3). Each value represents the mRNA level in the brain homogenate from one single mouse normalized by the mRNA level of HPRT in the same sample. Except for Oatp2, mRNA levels of other transporters in wild-type mouse brain were comparable ($p > 0.05$, one-way ANOVA followed by the Newman-Keuls multiple comparison test). The mRNA levels of Oatp2 were statistically different with those of other transporters ($p < 0.05$, one-way ANOVA followed by the Newman-Keuls multiple comparison test). The mRNA levels of all transporters in the brain of the Bcrp knockout mouse were similar to those in the brain of the wild-type mouse, except for the case of Bcrp. It is interesting that Mrp4 shows abundant expression in the brain homogenate, as much as Mdr1a. Furthermore, the concentration of Mrp4 mRNA was 4.7-fold greater in the BBB-enriched fraction than in the brain homogenate.

TABLE 3

The expression of Bcrp, Mdr1a, Mrp1, Mrp4, and Oatp2 in brain homogenate

All mRNA levels were measured by real-time quantitative PCR and normalized by the concentration of HPRT. These were results from independent cDNA samples from three wild-type and Bcrp knockout mice, respectively ($n = 3 \sim 4$, mean ± S.D., ratio to HRPT). BCRP KO and ND represent Bcrp knockout mice and not detected, respectively.

	Bcrp	Mdr1a	Mrp1	Mrp4	Oatp2
Wild-type	18.1 ± 2.3	26.9 ± 5.9	17.4 ± 1.1	27.7 ± 2.2	40.8 ± 7.6
Bcrp KO	ND	28.4 ± 3.3	19.1 ± 7.6	26.9 ± 1.9	44.2 ± 7.2

Discussion

In the present study, we examined the involvement of Bcrp in the efflux transport of DHEAS using the in situ brain perfusion method to examine the hypothesis that BCRP acts as a functional efflux transporter at the BBB for sulfoconjugated organic anions. In addition, the role of BCRP at the BBB was also investigated using another typical BCRP substrate, mitoxantrone.

Quantitative real-time PCR showed that the concentration of Bcrp mRNA in the brain was comparable with that of ABC transporters which are expressed in the mouse brain, such as Mdr1a, Mrp1, and Mrp4 (Table 3) and that Bcrp mRNA was enriched in the BBB-enriched fraction similar to Mdr1a P-gp mRNA. Western blot analysis revealed that Bcrp was clearly detectable in the BBB-enriched fraction, and the band density was greater in the BBB-enriched fraction than in the brain homogenate (Fig. 1A). Furthermore, immunohistochemical analysis revealed that Bcrp was localized at the luminal side of mouse brain capillaries (Fig. 1, B and C). All these results indicate that Bcrp is expressed and localized at the BBB, suggesting the possibility the Bcrp may play a role in the efflux of its substrates at the BBB.

Involvement of Bcrp in the transport of DHEAS and mitoxantrone at the BBB was investigated by examining the effect of GF120918 on their brain uptake determined using the in situ brain perfusion technique in the mouse. Because GF120918 inhibits both P-gp and Bcrp (Allen et al., 1999), the P-pg knockout mouse was used in conjunction with the wild-type mouse to exclude any confounding effects of P-gp inhibition (Jonker et al., 2000). Time-dependent brain uptake of [^{3}H]DHEAS was observed up to 2 min, and treatment with GF120918 increased the brain uptake of [^{3}H]DHEAS in a dose-dependent manner (Fig. 2), whereas it did not affect the distribution volume of sucrose (Table 2). This suggests that the effect of GF120918 is not due to a nonspecific effect, such as the destruction of the BBB by opening the tight junctions, but to inhibition of efflux transport at the BBB. Since GF120918 is an inhibitor of both P-gp and Bcrp (Allen et al., 1999), the brain uptake of [^{3}H]DHEAS was also determined in the Mdr1a/1b P-gp knockout mouse to exclude the possibility that the effect of GF120918 is due to inhibition of P-gp at the BBB (Fig. 3A). The brain uptake of [^{3}H]DHEAS in Mdr1a/1b P-gp knockout mice was comparable with that in wild-type mice, and the increased brain uptake of [^{3}H]DHEAS by GF120918 was still observed in Mdr1a/1b P-gp knockout mice (Fig. 3A). In the case of another typical substrate of Bcrp, mitoxantrone, GF120918 treatment increased the brain uptake of [^{3}H]mitoxantrone similar to that of [^{3}H]DHEAS (Fig. 3B). Since the brain uptake of [^{3}H]mitoxantrone was increased in Mdr1a/1b P-gp knockout mice compared with that in wild-type mice (Fig. 3B), the effect of GF120918 is partly accounted for by inhibition of P-gp. However, GF120918 was still effective in wild-type and Mdr1a/1b P-gp knockout mice (Fig. 3B). Therefore, in addition to P-gp, it is likely that a GF120918-sensitive transporter other than P-gp is involved in the efflux of mitoxantrone at the BBB. Recently, Cisternino et al. (2004) also reported that the brain uptake of mitoxantrone was linear up to 2 min using the in situ perfusion method, and its uptake was increased by treatment of GF120918. This is consistent with our results; although, there is a discrepancy in the effect of knockout of P-gp on the brain uptake of [^{3}H]mitoxantrone for some, as yet unknown, reason (Cisternino et al., 2004).

To show that the effect of GF120918 is due to inhibition of Bcrp, the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone was determined in Bcrp knockout mice (Fig. 4, A and B). Surprisingly, the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone was found to be comparable and independent of the Bcrp expression (Fig. 4, A and B). Furthermore, treatment with GF120918 still increased the brain uptake of [^{3}H]DHEAS even in Bcrp knockout mice (Fig. 4A). To examine the possibility of adaptive up- and/or down-regulation of transporters in the brain of Bcrp knockout mice, quantitative PCR was carried out. The mRNA levels of Mdr1a, Mrp1, Mrp4, and Oatp2 were similar to those in the brain of wild-type mice (Table 3). This suggests that distinct adaptive alteration of the expression of transporters may not have occurred in the brain of Bcrp knockout mice as far as these transporters are concerned. Taking all these results into consideration, especially the in situ analysis using Bcrp knockout mice, the contribution of Bcrp to the efflux transport of [^{3}H]DHEAS and [^{3}H]mitoxantrone at the mouse BBB was considered to be minor, if it exists at all, and thus it is suggested that other GF120918-sensitive transporter(s), distinct from Bcrp and P-gp, may account for the efflux of [^{3}H]DHEAS and [^{3}H]mitoxantrone at the BBB. Whether one and the same GF120918-sensitive efflux transporter affects [^{3}H]DHEAS and [^{3}H]mitoxantrone remains to be demonstrated.

Collectively, the present study could not demonstrate any involvement of Bcrp in the efflux transport of the Bcrp substrates, DHEAS and mitoxantrone, at the BBB, although Bcrp is abundantly expressed at the BBB and is likely to play an important role as a detoxification system in the central nervous system together with P-gp. This result is also supported by the recent findings of van Herwaarden et al. (2003). They reported that the hepatobiliary and intestinal elimination of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine was significantly reduced in Bcrp knockout mice; however, there was no significant change in the brain penetration between wild-type and Bcrp knockout mice. The functional role of Bcrp at the BBB remains virtually unknown. Mogi et al. (2003) reported that Akt signaling modulates the side population cell phenotype by regulating the translocation of Bcrp between the plasma membrane and intracellular compartment. We cannot exclude the possibility that the function of Bcrp at the BBB is also modulated by an unknown mechanism and works only under certain conditions. Further investigation is necessary to elucidate the role of Bcrp in the detoxification system in the brain. Cisternino et al. (2004) also demonstrated that the brain uptake of prazosin and mitoxantrone was increased by treatment with GF120918 in Mdr1a single knockout mice. It would be interesting to discover whether the effect of GF120918 on the brain uptake of prazosin is also ascribed to the inhibition of Bcrp-mediated efflux.

The present study shows the presence of a GF120918-sensitive efflux transporter for [^{3}H]DHEAS and [^{3}H]mitoxantrone at the BBB. The uptake transporter, Oatp2, is expressed in the luminal and abluminal membrane of the brain capillaries. According to the in situ study by Dagenais et al. (2001), the brain uptake of [D-penicillamine(2,5)]-enkephaline, a peptide substrate of Oatp2 in Mdr1a P-gp knockout

mice was saturable and inhibited by Oatp2 substrates, suggesting the involvement of Oatp2. Therefore, it is possible that Oatp2 accounts for the luminal uptake of DHEAS at the luminal membrane of brain capillaries as well as efflux from the brain at the abluminal membrane. Although the physiological meaning of luminal Oatp2 remains unknown, this uptake is also considered to be present in humans since the human isoform of Oatp2, OATP-A, has been shown to exhibit similar membrane localization in the brain capillaries (Gao et al., 1999, 2000). Thus, it may be important to limit Oatp2- and OATP-A-mediated DHEAS uptake by the GF120918-sensitive efflux transporter at the BBB in addition to facilitate the elimination of locally synthesized DHEAS from the brain to regulate the effect of DHEAS on neuronal function. In addition, the efflux transporter may account for the DHEAS compartmentalization between the brain and blood.

Currently no candidate transporter other than P-gp and Bcrp has been reported to interact with GF120918 (Hyafil et al., 1993; Allen et al., 1999; Evers et al., 2000). Interestingly, quantification of mRNA revealed the abundant expression of MRP4 (*ABCC4*), an ABC transporter classified as a member of the Mrp/MRP (*ABCC*) family, in the brain compared with other ABC transporters such as P-gp and Mrp1. Furthermore, Mrp4 mRNA was increased in the BBB-enriched fraction like Mdr1a and Bcrp. RT-PCR analyses have demonstrated its expression in primary cultured bovine brain capillary endothelial cells and the brain capillary-enriched fraction (Zhang et al., 2000). MRP4 shows broad substrate specificity for a number of compounds including DHEAS as well as cyclic nucleotides, methotrexate, estradiol-17β-glucuronide, and prostaglandins (van Aubel et al., 2002; Zelcer et al., 2003). In the revision process of this article, Leggas et al. (2004) reported that Mrp4 is localized at the luminal membrane of the brain capillaries and decreased the efflux rate of topotecan from the brain in Mrp4 knockout mice compared with wild-type mice. These recent results may support our speculation, and further investigation is necessary to show that Mrp4 is also involved in the efflux transport of organic anions at the BBB.

In conclusion, we have demonstrated that a GF120918-sensitive transporter(s) is involved in the efflux of [^{3}H]DHEAS and [^{3}H]mitoxantrone at the BBB, facilitating their elimination from the brain and limiting their uptake by the brain. Although BCRP is abundantly expressed in blood capillaries forming the BBB, we did not find any evidence indicating that Bcrp is a functionally active efflux transporter at the BBB.

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